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Biomarkers, Genomics, Proteomics, and Gene Regulation

Detection of Genomic Amplification of the Human Telomerase Gene *TERC,* a Potential Marker for Triage of Women with HPV-Positive, Abnormal Pap Smears

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The vast majority of invasive cervical carcinomas harbor additional copies of the chromosome arm 3q, resulting in genomic amplification of the human telomerase gene *TERC*. Here, we evaluated *TERC* amplification in routinely collected liquid based cytology (LBC) samples with histologically confirmed diagnoses. A set of 78 LBC samples from a Swedish patient cohort were analyzed with a four-color fluorescence *in situ* hybridization probe panel that included *TERC*. Clinical follow-up included additional histological evaluation and Pap smears. Human papillomavirus status was available for all cases. The correlation of cytology, *TERC* amplification, human papillomavirus typing, and histological diagnosis showed that infection with high-risk human papillomavirus was detected in 64% of the LBC samples with normal histopathology, in 65% of the cervical intraepithelial neoplasia (CIN)1, 95% of the CIN2, 96% of the CIN3 lesions, and all carcinomas. Seven percent of the lesions with normal histopathology were positive for *TERC* amplification, 24% of the CIN1, 64% of the CIN2, 91% of the CIN3 lesions, and 100% of invasive carcinomas. This demonstrates that detection of genomic amplification of *TERC* in LBC samples can identify patients with histopathologically confirmed CIN3 or cancer. Indeed, the proportion of *TERC*-positive cases increases with the severity of dysplasia. Among the markers tested, detection of *TERC* amplification in cytological samples has the highest combined sensitivity and specificity for discernment of low-grade from high-grade dysplasia and cancer. ***(Am J Pathol 2009, 175:1831–1847; DOI: 10.2353/ajpath.2009.090122)***

Cervical carcinoma is the second most common malignancy among women world-wide.[1] The introduction of screening programs based on cytological examination of cervical smears resulted in a significant decrease in both incidence and mortality rates.[2] The most common

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cervical cancer type is squamous cell cervical carcinoma.[3] Infections with high-risk human papillomavirus (HR-HPV) are almost invariably found in women with neoplastic disease and progression from low-grade to high-grade dysplasia, and invasive disease is very rare in the absence of HR-HPV.[4] However, transient HPV infections that do not result in the development of high-grade dysplasia are common in young, sexually active women.

The causal relationship between HR-HPV infection and cervical cancer has made the detection of the virus an attractive approach to identify women at risk of developing cervical cancer.[5] However, it has also become increasingly evident that other factors, in addition to HR-HPV per se, are required for cervical carcinogenesis, since only rarely will women infected with HR-HPV eventually develop cervical cancer. Therefore, biomarkers strongly associated with the propensity of low-grade lesions to progress to high-grade lesions and cancer would be of great value for the individualization of the clinical management of women with abnormal Pap smears.[6]

The expression of the HPV protein E7 triggers the activation of the cyclin-dependent kinase inhibitor $p16^{INK4a}$, which has been explored for its diagnostic potential.[7] In normal cells, p16 expression is essentially undetectable by immunocytochemistry. It is possible that such staining will not only improve the chances of detecting premalignant cells, but also aid in the distinction between premalignant and reactive atypia.[8]

In addition to infection with HPV and its consequent expression of p16, the majority of cervical carcinomas carry extra copies of the long arm of chromosome 3 and with it genomic amplification of the RNA component of the human telomerase gene (*TERC*), which resides on cytoband 3q26.[9,10] Therefore, genomic amplification of this gene is likely to play a central role in progression from low-grade dysplasia to high-grade cervical intraepithelial neoplasia (CIN) and invasive cancer. In a previous study, we showed that progression is never observed in the absence of genomic amplification, and, inversely, extra copies of this gene are not present in lesions that spontaneously regress.[11]

The aim of the present study was to validate these findings among women undergoing testing for cervical cancer following an abnormal Pap smear at population-based screening, and to correlate the amplification status of *TERC* with the histological evaluation of concordant biopsies. Genomic copy numbers of *TERC* and the oncogene *MYC* were determined on liquid based cytology (LBC) slides using fluorescence *in situ* hybridization (FISH), and the findings were systematically compared with HPV status, p16 protein expression, and the results of cytological screening and histopathological diagnoses.

Materials and Methods

Patients and Tissue Samples

We consecutively enrolled 78 women with any grade of cytological abnormalities detected at a population-based screening. The women were referred for extended testing at the Department of Gynecology at the Karolinska University Hospital Huddinge in Stockholm, Sweden. The extended testing took place during 2005, 2 to 6 months after the initial population-based screening. The mean age of the women was 35.3 years (median 33 years, range 23 to 60 years).

Clinical Evaluation

Enrollment, clinical characterization, and sampling of all women have been previously reported in detail.[12] From each woman we collected material for a Pap smear and LBC, and performed colposcopy and punch biopsies for histopathological evaluation. In many cases, a subsequent loop electrosurgical excision procedure (LEEP) histopathology and/or Pap smear follow-up were available.

Cytological and histopathological samples were re-evaluated by an experienced pathologist (A.H.). Samples were diagnosed in a blinded fashion, ie, the respective assessment was disclosed only after both the cytology and histology samples had been evaluated. Cytological categories were defined according to the Bethesda nomenclature,[13] including the subgroups within normal limits (WNL), atypical squamous cells—undetermined significance (ASCUS), low-grade squamous intraepithelial lesions (LSIL), atypical squamous cells—cannot exclude high-grade squamous intraepithelial lesions (HSIL) or high-grade changes (ASC-H), and HSIL. According to Swedish recommendations, cases of koilocytosis without signs of dysplasia are reported as WNL. Therefore, the LSIL group contains samples corresponding to cytological CIN grade 1 (CIN1) only. Histopathological diagnosis followed the World Health Organization criteria[14] and included the subclasses benign (WNL), CIN1, CIN2, CIN3, and carcinoma. Women with CIN2 or CIN3 were treated by LEEP.

Follow-up data were collected until 2007 (Table 1), including Pap smears in all cases and colposcopy, plus biopsy when indicated. LBC samples were used for all of the HPV analyses, the FISH analyses of *TERC*, *MYC*, and HPV, and for the p16 analysis. Additionally, for a subset of cases, 4-μm sections of the biopsies, and/or LEEPs were also hybridized and analyzed. All samples used in the study were obtained with informed consent and permission from the local ethical committee. The flow chart in Figure 1 summarizes the study design.

HPV Status

HPV status was evaluated by four different assays: 1) a prototype line blot analysis from Roche; 2) a quantitative real-time PCR method specific for 10 high-risk HPV types (HPV 16, 18/45 group, 31 and 33/35/39/52/58/67 group (Quantovir AB, Uppsala, Sweden); 3) detection of E6/E7 mRNA expression of HPV 16, 18, 31, 33, and 45 HR-HPV with real-time nucleic acid sequence-based amplification assay method (PreTect HPV-Proofer; NorChip AS, Oslo, Norway); and 4) HPV-FISH (see also Figure 1). The Roche line blot assay was performed as described earlier.[15–17] Details of the method and real-time PCR results obtained with the samples analyzed in this study, have been reported.[18,19] Results from the E6/E7 mRNA with the samples used in this communication have been described in a previous publication.[12] These previously published data were used only as reference points for performance comparison with the previously unpublished line blot and FISH data (Table 1 and Figure 2).[12,18,20]

In addition HPV was detected by FISH (see examples in Figure 3, A–E) as described below.

Status of p16 Expression

Results from previously published p16 immunocytochemical analyses[12] are summarized in Table 1. In short, p16 immunoreactivity was determined on LBC slides and scored as negative (−; less than three stained reactive cells per slide) or positive (+, ++, and +++) depending on the staining intensity.

FISH

Slides for FISH analyses were prepared from samples of LBC specimens according to standard procedures.[21] Four-color FISH analysis was performed on each case using a probe set targeting the centromere of chromosome 7 (CEP7), the *TERC* locus at chromosome band 3q26 (LSI TERC), the *MYC* locus at 8q24.2 (LSI MYC), and an HPV probe that contained DNA of the types 16, 18, 30, 45, 51, and 58. The probes were provided by Abbott Molecular, Inc. (Des Plaines, IL) through a Cooperative Research and Development Agreement (CRADA #001039). CEP7 was labeled with Spectrum Aqua, the *TERC* contig with Spectrum Gold, *MYC* with Spectrum Red, and the HPV probe was biotin-labeled. The HPV probe was previously shown to detect types 16, 18, 26, 31, 33, 35, 39, 45, 52, 53, 56, 58, 59, 66, and 82,[22,23] indicating that the DNA of the HPV types contained in the mix cross-reacts with other types because of sequence homology. There were no samples with HPV 30 or HPV51 infection included in the studies cited.[22,23]

Hybridization, posthybridization washes, and detection of the HPV probe were performed according to the probe manufacturer's recommendations.[22] In brief, the LBC slides were incubated for 2 minutes in 2× standard saline citrate at room temperature followed by pepsin (0.3 mg/ml in 10 mmol/L HCl) at 37°C for 5 to 10 minutes, fixed in 1% neutral-buffered formalin at room temperature for 5 minutes, and then washed in 1× PBS for 5 minutes. Slides were dehydrated in an ethanol series and air-dried. Co-denaturation at 72°C for 2 minutes and overnight hybridization at 37°C were done on a ThermoBrite (Statspin). Slides were then washed in 2× SSC/0.3% Nonidet P-40 for 2 minutes at 48°C and in 2× SSC/0.1% Nonidet P-40 for 1 minute at room temperature. The HPV probe was detected using Alexa Fluor 488 Tyramide Signal Amplification kit number 22 (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. Finally, antifade solution (VectaShield, Vector Laboratories, Burlingame, CA) containing the nuclear counterstain 4,6-diamidino-2-phenylindole was applied and coverslips were added.

Scoring of FISH Results

Image acquisition and analyses were performed using a Leica DM-RXA fluorescence microscope (Leica, Wetzlar, Germany) equipped with custom optical filters for 4,6-diamidino-2-phenylindole, Spectrum Aqua, Spectrum Gold, Spectrum Red, and Spectrum Green (Chroma Technologies, Rockingham, VT) and a ×40 Plan Apo (NA 1.25) objective. Scoring of FISH results was done without knowledge of cytological or histopathological evaluation or any of the other markers. HPV fluorescence patterns and *TERC* and *MYC* signals were enumerated by screening and counting the entire slide visually using the Spectrum Green filter for HPV, with the Spectrum Gold filter for *TERC*, and with the Spectrum Red filter for *MYC*. All nuclei on a slide were evaluated. On average, 2320 nuclei per slide with a range of 232 to 4996 nuclei were counted. For the HPV probe the results were classified as negative (0), or positive with episomal (1), episomal and integrated (2), or integrated (3) patterns depending on the appearance of the fluorescence signals (see Figure 3 as an example). CEP7, *TERC*, and *MYC* were evaluated by signal counts per nucleus. The results were registered as patterns for the probe panel (CEP7-*TERC*-*MYC*-HPV) for each nucleus. For example, a nucleus with two signals for CEP7, three signals for *TERC*, two signals for *MYC,* accompanied by an HPV-FISH episomal pattern was recorded as a "2321" pattern. Accordingly, cells with normal signal numbers and HPV-FISH negative result were reported as "2220." Their number was recorded on a manual counter and used to establish the total cell count by adding it to the number of nuclei with aberrant patterns. Nuclei with more than two *TERC* signals and/or more than two *MYC* signals and/or HPV-FISH positivity were imaged using a CoolSnap camera and multifocus imaging with the Leica CW4000-FISH imaging software for each of the optical filters. This software also allows for registering the imaged nuclei in relocation charts with their positions and their signal patterns. This enabled us to revisit the few cases for which ambiguous signal constellations were observed, and to arrive to a consensus count among three observers (P.S., K.H.-H., and T.R.). The complete FISH results comprised of the total cell count per slide and a list of all of the cells deviating from the FISH pattern of normal cells (see column "Signal patterns observed" in Table 1). All of the other columns provided for the FISH data in Table 1 were calculated from these raw data (eg, number of cells with more than two *TERC* signals excluding or including cells with four CEP7 and four *TERC* signals, etc). Cells with four signals for each probe, "444" were categorized as "tetraploid," acknowledging the fact that other chromosomal aneuploidies could exist in these cells.

Table 1. Comprehensive Table of All Analyses and Clinical Data for All 78 Cases Studied

	Case Number	Patient Age	Total Cells	TERC: Nuclei with >2 TERC signals, **including** nuclei with 4 signals for CEP7 and TERC	TERC: Nuclei with >2 TERC signals, **excluding** nuclei with 4 signals for CEP7 and TERC	MYC: Nuclei with >2 TERC signals, **including** nuclei with 4 signals for CEP7 and TERC	**Tetraploid nuclei (444 patterns)**	HPV-FISH positive cells with >2 signals for TERC and/or MYC (double positive nuclei)	**Signal patterns observed** — Patterns observed: First digit:CEP7 signals, second digit: TERC signals, third digit: MYC signals,fourth digit: HPV pattern (O=neg, 1=episomal,2= episomal and integrated, 3=integrated, ?=not analyzable) x number of cells observed with this specific pattern
Highest grade histology: WNL lesions (n=14)	3	45	1205	0	0	0	0	0	222?x1205
	130	26	2032	0	0	2	0	0	2230x1,2240x1(possibility of doublets),2221x2, 2220x2028
	179	27	1188	0	0	0	0	0	2220x1188
	246	30	1678	0	0	0	0	0	2220x1678
	82	47	3002	1	1	0	0	0	?42?x1,222?x3001
	85	33	3364	1	1	1	0	0	2221x21,2223x2,?221x1,??21x1,22(1df,1d)2(1d,1)0x1,
	159	24	2883	1	1	1	0	0	222?x2,2440x1,2220x2880
	222	33	2367	1	1	0	0	0	2223x3,2420x1,2220x2363
	11	50	861	1	1	1	0	1	2443x1,2220x860
	228	25	3320	2	1	2	1	0	23(?)3(?)0x1,4440x1,2220x3318
	167	25	2210	2	1	5	1	0	2223x1,2221x3,2240x1,2230x2,2360x1,4440x1, 2220x2201
	145	47	994	4	0	4	4	0	2221x1,4440x4,2220x989
	96	49	2637	6	5	4	0	0	2221x4,2222x6,2223x2,222?x2,2420x2,2440x2,4450x1, 5550x1,2220x2617
	182	**57**	**1815**	**28**	**3**	**28**	**4**	**8**	**2223x2,23or44or53x2,3450x1,4440x4,4450x14,4451x2, 4453x4,445?x1,???3x1,2220x1785**
Highest grade histology: CIN1 lesions (n=17)	132	36	496	0	0	0	0	0	2221x1,2222x3,2220x492
	180	27	1977	0	0	0	0	0	2220x1977
	215	28	1193	0	0	1	0	1	0001x1,2221x9,2223x2,223(?)(1df,1)1x1,2220x1180
	248	42	4006	1	0	1	1	0	2221x16,2223x1,4440x1,2220x3988
	86	30	1032	1	1	0	0	0	2221x3,2222x2,2223x5,2320x1,2220x1021
	6	36	535	1	1	1	0	1	0222x2,2221x1,2222x4,3431x1,2220x527
	224	51	2903	2	2	0	0	0	2320x1,2420x1,2220x2901
	187	24	2530	3	0	3	3	0	4440x3,2220x2527
	209	24	2099	4	3	3	0	1	2420x1,?440x1,3440x1,2221x4,3443x1,2220x2091
	76	46	2708	6	4	5	2	1	?213x1,2221x3,2222x1,2223x7,2320x1,2440x1,2443x1, 29(6ds)60x1,4440x2,2220x2690
	193	40	833	7	0	7	7	0	4440x6,2or4440x1,2220x826
	223	24	865	7	1	7	4	5	2221x3,2222x2,4440x1,4441x3,3or43or43or41x1,4840x 1, 4(?)442x1,2220x853
	142	30	4996	8	3	8	0	1	2221x1,2223x2,4440x2,2or4440x1,2or4443x1, 3or4440x1,3440x3,2220x4985
	220	**29**	**2877**	**10**	**9**	**7**	**1**	**0**	**2320x3,2430x1,2440x1,3850x2,3440x2,4440x1, 2220x2867**
	128	**23**	**2882**	**14**	**0**	**14**	**14**	**2**	**2221x2,2223(?)x1,22or321x1,4440x13,43or441x1, 2220x2864**
	160	**27**	**2701**	**18**	**13**	**16**	**5**	**1**	**2223x1,2420x1,2430x1,3420x1,3430x10,4440x4, 4443x1,2220x2682**
	173	**40**	**3727**	**42**	**9**	**42**	**33**	**0**	**444?x33,454?x3,466?x2,465or6?x4,222?x3685**

(*table continues*)

Table 1. *Continued*

Cytology/Biopsy Histology				Follow-Up (LEEP Histology, subsequent Pap smear, Biopsies and LEEPs)			"Highest Grade Histology"	HPV testing						p16
Referral clinic Pap smear just prior to repeat testing	Repeat Testing			LEEP Histology	Time between Biopsy and LEEP in month	Other follow-ups until 2007 addition to the LEEP		HPV FISH positive nuclei		Line blot		QuantoVir	NorChip	
	Pap smear cytology	Thinprep cytology	Biopsy Histology					Total No.	No. integr	High Risk	Low Risk			
HSIL	LSIL	WNL	WNL	no LEEP	n.a.	WNL Pap smears	WNL	n.d.	0	73	54,61, 62,81	16	0	0
LSIL	WNL	WNL	WNL	no LEEP	n.a.	WNL Pap smears	WNL	2	0	68	0	0	0	0
LSIL	LSIL	LSIL	WNL	no LEEP	n.a.	2005 Pap inflamm., bx WNL, 2007 WNL Pap smear	WNL	0	0	0	83	0	0	0
control after LEEP	WNL	LSIL	WNL	no LEEP	n.a.	WNL Pap smears	WNL	0	0	0	62	n.d.	n.d.	0
LSIL	LSIL	LSIL	WNL	no LEEP	n.a.	WNL Pap smears	WNL	n.d.	n.d.	0	54,61, 81	16	0	0
LSIL	WNL	LSIL	WNL	no LEEP	n.a.	2005 Pap LSIL, bx WNL 2006, WNL Pap	WNL	25	2	58	54,61	33	33	0
LSIL	WNL	LSIL	WNL	no LEEP	n.a.	WNL Pap smears	WNL	0	0	39	84,61, 6108	39	0	0
LSIL	LSIL	LSIL	WNL	no LEEP	n.a.	2005 Pap smear WNL, bx CIN1, 2006 & 2007 WNL Pap smears	WNL	3	3	52	61,62	33, 31	0	0
LSIL	WNL	WNL	WNL	no LEEP	n.a.	no follow-up	WNL	1	1	0	0	45	0	diff.
LSIL	n.d.	HSIL	WNL	no LEEP	n.a.	WNL Pap smears	WNL	0	0	0	0	0	0	0
LSIL	LSIL	WNL	WNL	no LEEP	n.a.	2005 Pap WNL, bx CIN1, 2006 & 2007 WNL Pap and bx	WNL	4	1	16, 39	0	16, 39	0	1+
LSIL	LSIL	LSIL	WNL	no LEEP	n.a.	no follow-up	WNL	1	0	56	0	39	0	0
LSIL	LSIL	LSIL	WNL	inflamm	12	2005 Pap+bx CIN1, 2006 Pap LSIL	WNL	12	2	53,56	54	0	0	0
ASCUS	**ASC-H**	**HSIL**	**WNL**	**WNL**	**2**	**after LEEP WNL Pap smears**	**WNL**	**11**	**9**	**58**	**0**	**33**	**0**	**2+**
LSIL	LSIL	WNL	CIN1	no LEEP	n.a.	WNL	CIN1	4	0	0	0	0	0	0
LSIL	LSIL	LSIL	CIN1	CIN1	24	after LEEP WNL Pap+bx	CIN1	0	0	56	0	0	0	0
LSIL	LSIL	LSIL	CIN1	no LEEP	n.a.	2006 WNL Pap smear	CIN1	13	2	16	0	16	16	0
LSIL	LSIL	LSIL	CIN1	no LEEP	n.a.	2006 WNL Pap	CIN1	17	1	18	0	n.d.	n.d.	0
ASCUS	WNL	WNL	WNL	CIN1	12	after LEEP WNL Pap	CIN1	10	5	0	62,72	n.d.	n.d.	0
ASCUS	LSIL	WNL	CIN1	WNL	36	after LEEP WNL Pap	CIN1	8	0	16	0	0	0	0
ASCUS	n.d.	LSIL	CIN1	CIN1	0	after LEEP: 2005 Pap WNL 2006 bx CIN1, LEEP, CIN1	CIN1	0	0	0	0	0	0	0
ASCUS	ASC-H	ASCUS	CIN1	WNL	5	2008 Pap ACIS?,bx WNL	CIN1	0	0	0	0	0	0	0
LSIL	LSIL	LSIL	CIN1	no LEEP	n.a.	2006 WNL Pap+bx	CIN1	5	1	39,45	81	18,45	45	0
LSIL	LSIL	LSIL	CIN1	CIN1	3	after LEEP WNL Pap smears	CIN1	13	9	0	62,72 84,IS	0	0	0
ASC-H	ASC-H	WNL	CIN1	CIN1	2	after LEEP WNL Pap	CIN1	0	0	0	0	0	0	?
LSIL	LSIL	LSIL	WNL	CIN1	10	after LEEP 2005,2. LEEP in 2006 CIN1, WNL Pap after	CIN1	10	0	16	0	16	16	0
HSIL	ASCUS	LSIL	CIN1	no LEEP	n.a.	no follow-up	CIN1	4	3	18,16	0	16,18 31	18	1+
LSIL	**HSIL**	**HSIL**	**WNL**	**CIN1**	**3**	**after LEEP WNL Pap**	**CIN1**	**0**	**0**	**31**	**0**	**31**	**0**	**1+**
HSIL	**LSIL**	**LSIL**	**CIN1**	**no LEEP**	**n.a.**	**WNL Pap smears**	**CIN1**	**5**	**1?**	**51,66**	**54, 6108**	**0**	**0**	**0**
LSIL	**AGUS**	**LSIL**	**CIN1**	**no LEEP**	**n.a.**	**WNL Pap smears**	**CIN1**	**2**	**2**	**16,73**	**62, 6108**	**16**	**16**	**0**
ASCUS	**ASC-H**	**LSIL**	**CIN1**	**CIN1**	**0.5**	**after LEEP WNL Pap**	**CIN1**	**n.d.**	**n.d.**	**31**	**0**	**31**	**0**	**0**

(*table continues*)

Table 1. *Continued*

	Case Number	Patient Age	Total Cells	TERC: Nuclei with >2 TERC signals, **including** nuclei with 4 signals for CEP7 and TERC	TERC: Nuclei with >2 TERC signals, **excluding** nuclei with 4 signals for CEP7 and TERC	MYC: Nuclei with >2 TERC signals, **including** nuclei with 4 signals for CEP7 and TERC	**Tetraploid nuclei (444 patterns)**	HPV-FISH positive cells with >2 signals for TERC and/or MYC (double positive nuclei)	Signal patterns observed: Patterns observed: First digit:CEP7 signals, second digit: TERC signals, third digit: MYC signals,fourth digit: HPV pattern (O=neg, 1=episomal,2= episomal and integrated, 3=integrated, ?=not analyzable) x number of cells observed with this specific pattern
Highest grade histology: CIN2 lesions (n=22)	104	36	526	0	0	0	0	0	2220x526
	146	23	3878	0	0	0	0	0	2221x4,2222x3,2223x9,2220x3862
	152	27	777	1	1	1	0	1	2221x9,2222x1,2442x1,222?x1,2220x765
	121	23	2000	2	1	1	1	0	2221x6,2222x5,2223x1,2420x1,4440x1,2220x1986
	200	38	2544	2	2	1	0	0	?570x1,2320x1,2220x2542
	244	30	796	3	3	1	0	0	2221x3,2222x3,2223x2,2320x2,43(?)30x1,2220x785
	94	43	2869	4	1	4	3	0	2221x1,4440x3,6660x1,2220x2864
	192	31	2566	6	5	1	1	0	2320x5, 4440x1,2220x2560
	190	**58**	**655**	**9**	**9**	**7**	**0**	**0**	**2-4(doublets)4(doublets)0x3, 2-3(split d)20x1, 2-3(split d)3(splitd)0x1, 2-4(doublets)2(doublets)0x1, 2-4(doublets)4-8(doublets)0x1, 2(big)4-8(doublets)4-8(doublets)0x1(Endoreduplication?), 2-4 8(doublets)4(doublets)0x1(Endoreduplication?),2220x646**
	126	**28**	**2694**	**10**	**3**	**8**	**7**	**2**	**2221x5,2222x4,2223x4,2421x2,22(?)2(?)1x1,4440x7, 8880x1,2220x2670**
	124	**34**	**2330**	**12**	**1**	**11**	**11**	**2**	**2320x1,4440x9,4443x2,2220x2318**
	191	**31**	**2605**	**12**	**2**	**11**	**10**	**0**	**2320x1,3440x1, 4440x10, 2220x2593**
	134	**30**	**1313**	**13**	**7**	**15**	**5**	**6**	**2221x6,2222x3,2223x8,2232x1,2233x2,2322x1,4440x2, 4441x1,4443x1,444?x1,4550x6,?44?x1,2220x1280**
	235	**31**	**3013**	**14**	**0**	**12**	**11**	**3**	**2221x2,2222x3,2223x7,4440x8,4442x1,4443x1,444?x1, 4450x2,444or51x1,2220x2987**
	219	**58**	**2914**	**20**	**16**	**20**	**4**	**0**	**2340x6,2440x10,4440x4,2220x2894**
	214	**38**	**1111**	**24**	**21**	**20**	**3**	**3**	**2330x3,2333x2,2420x3,2430x3,2530x1,2550x1,2620x1, 2880x2,21080x1,21163x1,3430x1,3440x2,4440x3, 2220x1087**
	157	**23**	**3049**	**31**	**1**	**30**	**30**	**3**	**2221x18,2222x6,2223x7,2320x1,22(?)2(?)0x1,4440x27, 4441x2,4443x1,2220x2986**
	226	**28**	**3151**	**36**	**36**	**8**	**0**	**0**	**2320x7,2420x21,4640x8,2220x3115**
	103	**30**	**2112**	**46**	**2**	**45**	**44**	**4**	**2320x1,4440x40,4443x4,4680x1,2220x2066**
	177	**60**	**4021**	**59**	**59**	**58**	**0**	**0**	**2540x1,3630x1,41550x1,41570x1,41230x1,41040x1, 4530x1,4620x1,4630x6,4730x4,4830x32,4840x2, 4940x1,61570x2,61260x1,71460x2,71470x1,2220x3962**
	116	**34**	**4091**	**62**	**6**	**57**	**56**	**2**	**2223x2,2320x5,3330x1,4440x53,4442x1,4443x1, 444?x1,2220x4027**
	249	**31**	**2142**	**74**	**72**	**69**	**2**	**0**	**2430x1,2440x2,2550x1,3350x1,3440x57,3450x1, 34?0x5,3550x1,34or540x1,4440x2,46- 740x2,2220x2068**
CIN3 (n=23)	118	24	4671	0	0	0	0	0	2222x1,???1or2x2,2220x4668
	230	27	3954	4	2	4	2	3	2221x64,2222x13,2223x7,2442x1,4440x1,4441x1,45or6 8(?)2x1,2220x3866
	242	**27**	**984**	**9**	**0**	**9**	**8**	**8**	**2221x5,4440x1,4441x3,4442x1,4443x3,443or41x1,222 0x970**
	181	**52**	**2263**	**9**	**8**	**9**	**1**	**3**	**2221x19,2222x10,2223x1,2330x3,2320x2,2322x1, 2230x2,3781x1,4440x1,3330x1,2231x1,2220x2221**
	144	**55**	**232**	**15**	**15**	**4**	**0**	**0**	**232?x8,233?x1,242?x2,484?x2,2or332?x1,687?x1, 222?x217**
	240	**39**	**786**	**16**	**9**	**12**	**3**	**1**	**0001x1,2221x24,2222x7,2223x12,222?x2,2320x3, 2322x1,2440x1,3440x3,4440x2,444?x1, 4770(2330and2440)x1,44or64or50x1,2or4443(?)x1, 3or4430x1,3or4440x1,2220x724**

(*table continues*)

Table 1. *Continued*

Cytology/Biopsy Histology	Repeat Testing			Follow-Up (LEEP Histology, subsequent Pap smear, Biopsies and LEEPs)			"Highest Grade Histology"	HPV testing						p16
Referral clinic Pap smear just prior to repeat testing	Pap smear cytology	Thinprep cytology	Biopsy Histology	LEEP Histology	Time between Biopsy and LEEP in month	Other follow-ups until 2007 addition to the LEEP		HPV FISH positive nuclei: Total No.	HPV FISH positive nuclei: No. integr	Line blot: High Risk	Line blot: Low Risk	QuantoVir	NorChip	
LSIL	LSIL	LSIL/HSIL	CIN2	CIN1	12	after LEEP WNL Pap smears	CIN2	0	0	31	0	0	0	0
LSIL	WNL	LSIL	CIN1	CIN2	17	after LEEP WNL Pap smears	CIN2	16	9	16,58	0	16,33	16	0
LSIL	LSIL	LSIL	CIN2	CIN1	1	after LEEP WNL Pap in 2005	CIN2	11	0	56	0	0	0	1+
HSIL	HSIL	HSIL	CIN2	CIN2	2.5	after LEEP WNL Pap smears	CIN2	12	1	31,52, 67	0	31	0	2+
LSIL	WNL	HSIL	CIN2	CIN2	3	after LEEP WNL Pap smears	CIN2	0	0	68	0	39	0	0
HSIL	ASC-H	HSIL	CIN2	CIN1	4	after LEEP WNL Pap smears	CIN2	8	2	16,33, 58	42,61	n.d.	n.d.	3+
HSIL	LSIL	LSIL/HSIL	CIN1	CIN2	3	2005+2006 Pap and bx WNL, 2007 Pap LSIL, bx CIN1, LEEP CIN1	CIN2	1	0	31,45, 73	0	45	45	2+
ASC-H	WNL	HSIL	CIN2	CIN2	2.5	after LEEP WNL Pap	CIN2	0	0	33,39	0	33	0	2+
ASCUS	**ASC-H**	**ASC-H**	**CIN2**	**CIN2**	**3**	**after LEEP WNL Pap smears and bx**	**CIN2**	**0**	**0**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**0**
HSIL	**HSIL**	**LSIL**	**CIN2**	**CIN2**	**2**	**after LEEP Pap smear LSIL in 2005, WNL Pap in 2006 and 2007**	**CIN2**	**16**	**4**	**56,51**	**0**	**0**	**0**	**0**
LSIL	**HSIL**	**HSIL**	**CIN2**	**CIN2**	**1.5**	**after LEEP WNL Pap smears**	**CIN2**	**2**	**2**	**31**	**0**	**0**	**0**	**1+**
ASC-H	**WNL**	**WNL**	**CIN1**	**CIN2**	**1**	**after LEEP WNL Pap smears**	**CIN2**	**0**	**0**	**0**	**70**	**0**	**0**	**0**
LSIL	**HSIL**	**LSIL**	**CIN2**	**CIN2**	**2.5**	**after LEEP WNL Pap smears**	**CIN2**	**23**	**11**	**16,18**	**0**	**16,18**	**16**	**0**
LSIL	**HSIL**	**HSIL**	**CIN2**	**CIN2**	**2.5**	**after LEEP WNL Pap smears**	**CIN2**	**15**	**8**	**16,18, 31**	**0**	**18,16, 31**	**18**	**3+**
HSIL	**ASC-H**	**HSIL**	**CIN2**	**CIN2**	**2**	**after LEEP WNL Pap smears**	**CIN2**	**0**	**0**	**31**	**0**	**16**	**0**	**2+**
HSIL	**HSIL**	**HSIL**	**CIN2**	**CIN2**	**1.5**	**2007 Pap HSIL, bx inflammation**	**CIN2**	**3**	**3**	**56**	**0**	**0**	**0**	**0**
HSIL	**ASCUS**	**ASCUS**	**CIN1**	**CIN2**	**14**	**after LEEP WNL Pap smears**	**CIN2**	**34**	**8**	**16,51 66**	**0**	**16**	**16**	**0**
HSIL	**n.d.**	**HSIL**	**CIN2**	**CIN2**	**2**	**after LEEP WNL Pap smears**	**CIN2**	**0**	**0**	**31**	**0**	**31**	**0**	**1+**
ASCUS	**LSIL**	**LSIL/HSIL**	**CIN2**	**CIN2**	**1**	**after LEEP WNL Pap smears**	**CIN2**	**4**	**4**	**16**	**0**	**16**	**0**	**0**
LSIL	**LSIL**	**HSIL**	**CIN2**	**CIN2**	**3**	**2005 hysterectomy CIN1, no follow-up after**	**CIN2**	**0**	**0**	**66**	**0**	**0**	**0**	**1+**
LSIL	**HSIL**	**HSIL**	**CIN2**	**CIN2**	**1.5**	**after LEEP WNL Pap smears**	**CIN2**	**4**	**3**	**31**	**81**	**31**	**0**	**1+**
LSIL	**ASC-H**	**HSIL**	**CIN2**	**CIN2**	**3**	**after LEEP WNL Pap**	**CIN2**	**0**	**0**	**31**	**0**	**n.d.**	**n.d.**	**1+, 2+**
HSIL	WNL	LSIL	CIN1	CIN3	6	after LEEP WNL Pap smears	CIN3	3	0	33,59	84	31	0	1+
ASC-H	n.d.	LSIL	CIN3	CIN1	1	no follow-up after LEEP	CIN3	87	7	16	0	16	16	2+
HSIL	**HSIL**	**HSIL**	**CIN3**	**CIN3**	**3**	**after LEEP WNL Pap smears**	**CIN3**	**13**	**3**	**16**	**0**	**n.d.**	**n.d.**	**0**
HSIL	**HSIL**	**HSIL**	**CIN3**	**CIN3**	**2**	**2005 and 2006 WNL Pap, 2007 Pap inflammation**	**CIN3**	**33**	**11**	**16**	**0**	**16**	**16**	**3+**
ASC-H	**HSIL**	**HSIL**	**CIN3**	**CIN3**	**1.5**	**2005 hysterect. CIN1, WNL Pap smears after**	**CIN3**	**n.d.**	**n.d.**	**16**	**0**	**16**	**16**	**0**
ASC-H	**HSIL**	**HSIL**	**CIN3**	**CIN3**	**1**	**after LEEP WNL Pap smears**	**CIN3**	**46**	**13**	**16,18**	**0**	**16**	**16**	**2+**

(*table continues*)

Table 1. *Continued*

	Case Number	Patient Age	Total Cells	TERC		MYC	Tetraploid nuclei (444 patterns)	HPV-FISH positive cells with >2 signals for TERC and/or MYC (double positive nuclei)	Signal patterns observed
				Nuclei with >2 TERC signals, **including** nuclei with 4 signals for CEP7 and TERC	Nuclei with >2 TERC signals, **excluding** nuclei with 4 signals for CEP7 and TERC	Nuclei with >2 TERC signals, **including** nuclei with 4 signals for CEP7 and TERC			Patterns observed: First digit:CEP7 signals, second digit: TERC signals, third digit: MYC signals,fourth digit: HPV pattern (O=neg, 1=episomal,2= episomal and integrated, 3=integrated, ?=not analyzable) x number of cells observed with this specific pattern
Highest grade histology: CIN3 lesions (n=23)	225	33	2993	19	8	18	11	0	2440x7,4440x11,4520x1,?22?x1,2220x2973
	233	34	3749	20	9	64	5	0	223?x41,223?x2,224?x1,233?x1,254?x1,23or43?x1, 444?x5,445?x2,443or4?x1,545?x6,4or545?x3, 222?x3685
	112	30	4882	20	19	1	1	13	0001x1,2221x1,2320x2,2321x8,2322x1,2323x1,2420x4, 2421x3,4440x1,2220x4860
	120	37	1121	21	19	20	1	0	1420x1,1530x15,2440x1,2530x2,4440x1,3(?)440x1, 2220x1100
	204	42	2251	25	21	20	2	0	?44?x2,232?x4,233?x2,242?x1,243?x2,244?x4,334?x1, 364?x6,375?x1,444?x2,222?x2226
	208	40	3714	29	1	29	28	5	21(?)23x1,2221x2,2222x1,2223x38,222?x1,2443x1, 4440x24,4443x4,2220x3642
	206	28	2140	40	24	34	15	5	2320x6,3430x3,3440x3,3450x1,3530x2,31283x1, 3770x1,3773x1,4350x1,4431x1,4440x15,4550x1, 4552x1,4770x1,5472x1.6760x1,2220x2100
	17	25	2157	41	34	36	7	7	2320x1,2330x2,2420x2,2430x1,2431x1,2432x1,2440x5, 244?x1,2460x1,2520x1,2530x1,253?x1,2540x2,2640x2, 2642x1,2742x12753x1,3520x1,3540x1,3742x1,4440x5, 4442x1,444?x1,4540x1,4630x1,4640x2,464?x2, 2220x2116
	16	28	2073	49	49	48	0	0	2330x3,2420x1,2430x45,2220x2024
	232	38	3475	51	8	54	31	24	2221x755,2222x228,2223x82,2320x2,2321x3, 343or41x1,4421x2,4433x1,4440x18,4441x5,4442x2, 4443x6,4450x4,4453x3,445?x1,4543x1,33or450x1, 2220x2360
	201	45	2620	51	40	16	11	0	2320x14,2330x1,2420x21,2430x1,3530x1,4440x11, 4530x2,2220x2569
	111	40	3151	61	61	101	0	87	2211x2,2212x1,2221x13,2222x5,2230x3,2231x28, 2232x7,2240x2,2241x1,2242x3,2251x2,2261x2,2321x2, 2330x1,2331x5,2332x2,2340x2,2341x2,2351x1,2352x1, 2420x3,2421x3,2430x6,2431x2,2432x5,2440x1,2441x1, 2442x2,2450x1,2451x1,2452x1,2462x1,2530x1,2531x3, 2541x1,2542x3,2543x2,2552x1,2563x1,2740x1,2742x2, 4650x1,4863x1,48103x1,2220x3021
	250	35	1393	70	69	21	1	23	2222x1,2320x6,2323x2,2420x27,2423x14,2443x2, 2533x1,3330x1,34or543x1,4440x1,4540x1,4840x8, 4843x3,48(?)40x3,2220x1322
	90	26	2008	109	109	45	0	75	2221x20,2222x5,2231x7,2232x1,2241x3,2320x23, 2321x37,2330x1,2331x6,2332x3,2341x3,2421x3, 2430x3,2431x4,2432x4,2440x8,2442x1,2521x2, 252?x10, 2541x1,2220x1863
	227	28	3403	133	133	133	0	0	2221x8,3640x123, 364?x 8,1-4 6 3-4 0x2,2220x3262
	139	35	2002	151	137	147	1	30	2333x1,2423x1,2430x1,2430x5,2432x1,2533x1,2660x1, 3330x3,3332x1,3420x1,3423x1,3430x92,3431x1, 3432x4,3433x8,3440x1,3530x1,3880x1,4423x1, 4430x6,4433x6,4440x1,4530x1,4562x1,4750x1,4760x2, 4860x1,5533x1,5650x1,5860x1,6560x1,7993x1, 89103x1,2220x1851
	207	36	2224	35	34	34	1	0	2340x1,2430x4,2530x1,3430x22,3530x3,3740x1, 4440x1,4850x1,3420x1,2220x2189
Cancer (n=2)	113	35	4566	2992	2992	95	0	20	2320x2,2420x6,2520x2869,2523x20,47or840x2, 4740x25,4840x12,48or1040x1,41040x55,2220x1574
	156	39	1436	20	20	1	0	0	2223x1,2320x13,232or30x3,232or40x2,2440x1,3420x1, 2220x1415

(*table continues*)

Table 1. *Continued*

Cytology/Biopsy Histology				Follow-Up (LEEP Histology, subsequent Pap smear, Biopsies and LEEPs)			"Highest Grade Histology"	HPV testing						p16
Referral clinic Pap smear just prior to repeat testing	Repeat Testing: Pap smear cytology	Repeat Testing: Thinprep cytology	Repeat Testing: Biopsy Histology	LEEP Histology	Time between Biopsy and LEEP in month	Other follow-ups until 2007 addition to the LEEP		HPV FISH positive nuclei: Total No.	HPV FISH positive nuclei: No. integr	Line blot: High Risk	Line blot: Low Risk	QuantoVir	NorChip	
HSIL	n.d.	HSIL	CIN3	CIN3	0	2005 Pap WNL, no f/u for 2006-7	CIN3	0	0	0	6	0	0	0
LSIL	HSIL	HSIL	CIN3	CIN3	1	after LEEP WNL Pap smears	CIN3	n.d.	n.d.	68	0	39	0	2+
LSIL	WNL	LSIL	CIN1	CIN3	17	after LEEP WNL Pap smears	CIN3	15	1	16	0	16	16	1+
ASCUS	SQ CA	AD CA	CIN3	CIN3	1	after LEEP WNL Pap smears	CIN3	0	0	31	0	0	0	1+
ASCUS	ASC-H	ASC-H	CIN1	CIN3	1	2006 Pap smear LSIL, 2007 bx CIN1, LEEP CIN1	CIN3	n.d.	n.d.	16,53	0	16	16	1+
n.d.	WNL	WNL	CIN3	CIN3	1.5	after LEEP WNL Pap smears	CIN3	47	44	16	0	n.d.	n.d.	3+
ASCUS	HSIL	HSIL	CIN3	CIN2-3	2	after LEEP WNL Pap smears	CIN3	5	2	16,51	0	16	16	2+
HSIL	HSIL	HSIL	CIN3	CIN3	1.5	after LEEP two WNL Pap in 2004, no further follow-up	CIN3	7	1	16,33, 56	0	16,31 33	16, 33	2+
ASCUS	ADCA	HSIL	CIN3	CIN3	1	after LEEP WNL Pap smears	CIN3	0	0	73	0	0	0	3+
HSIL	HSIL	HSIL	CIN3	CIN3	0.5	after LEEP WNL Pap smears	CIN3	1089	93	16,51	0	16	16	0
HSIL	ASCUS	LSIL	CIN3	CIN3	1	after LEEP WNL Pap smears	CIN3	0	0	51	81,62	0	0	1+
HSIL	HSIL	HSIL	CIN3	CIN3	1	after LEEP WNL Pap smears	CIN3	108	5	16	0	16	16	3+
HSIL	ASC-H	HSIL	CIN2	CIN3	2	after LEEP WNL Pap smears	CIN3	24	23	16	0	n.d.	n.d.	1+ 2+
HSIL	HSIL	HSIL	CIN3	CIN3	2	Apr05 Pap WNL bx CIN1, Dec05 Pap WNL bx CIN2 Apr06 Pap WNL bx CIN1 Dec06,Feb08 Pap WNL bx insuf	CIN3	100	0	16	0	16	16	2+
HSIL	n.d.	HSIL	CIN3	CIN3	0	after LEEP 2005 Pap LSIL, 2006,2007 WNL Pap	CIN3	8	0	45, 52,68	0	16,33	45	3+
ASCUS	ASCUS	HSIL	CIN3	CIN3	10	after LEEP WNL Pap smears	CIN3	30	22	18	0	18	18	2+
LSIL	ASC-H	ASC-H	WNL	ACIS	6	after LEEP WNL Pap smears	ACIS (ca in situ)	0	0	18	0	n.d.	n.d.	1+
LSIL	SQ Ca	SQ Ca	SQ Ca	inv.cancer	1	after Wertheim surg. No follow-up	inv. cancer	20	20	16	0	16	16	3+
ASCUS	ASCUS	WNL	CIN3	microinv. cancer	2.5	2005 hysterect. CIN1, WNL Pap after	micro. inv.ca	1	1	16	0	16,39	16	0

*Bold text indicates: *TERC* positive according to threshold of nine and more nuclei with more than 2 *TERC* signals.

Abbreviations: n.a. (not applicable); n.d. (not determined); bx (biopsy); d (doublet); df (doublet far apart); ds (doublets); diff (diffuse); WNL (within normal limits); ASCUS (atypical squamous cells—undetermined significance); LSIL (low-grade squamous intraepithelial lesions); ASC-H (atypical squamous cells—cannot exclude high-grade squamous intraepithelial lesions (HSIL) or high-grade changes; HSIL (high-grade squamous intraepithelial lesions; (LEEP) loop electrosurgical excision procedure.

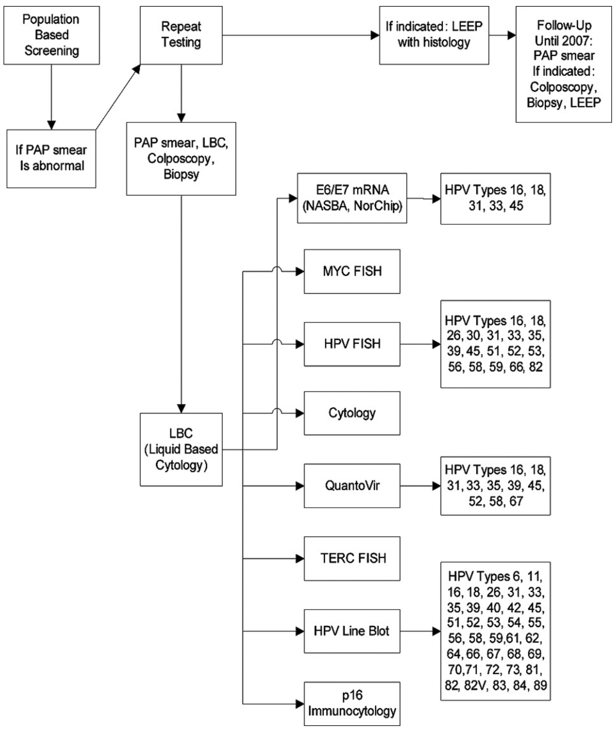


Figure 1. Flow chart of the study design.

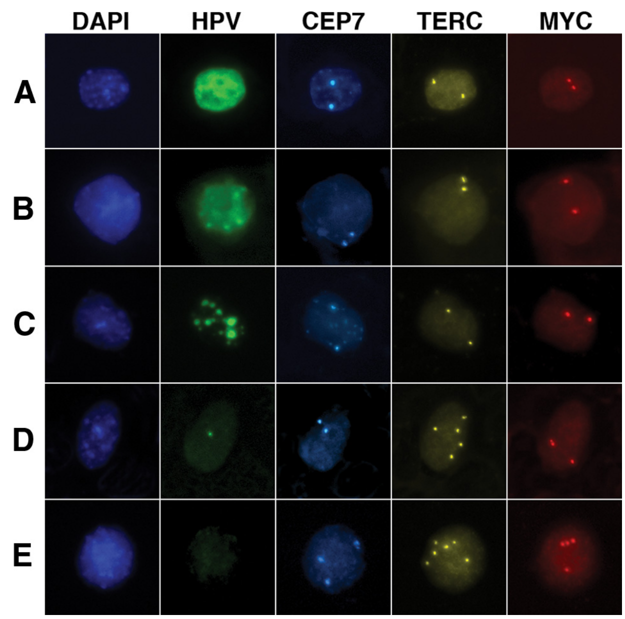


Figure 3. Examples for HPV-positive (**A, B, C, D**) and negative (**E**) nuclei, with and without chromosomal aberrations. The images show the 4,6-diamidino-2-phenylindole staining of the cell nucleus (Blue), the HPV staining (Green) in infected cells, the CEP7 probe (Aqua), the *TERC* probe (Gold), and the *MYC* probe (Red). Nucleus **A** represents a cell episomally infected with HPV (see *Material and Methods*: pattern 1), with two normal signals for each probe; **B** shows a cell with a mixed infection (episomal and integrated: pattern 2) with normal signals for all probes; and **C** displays an integrated HPV infection (pattern 3), also still normal for all probes. Nucleus **D** also shows an integrated HPV infection (only one integration site) with a diploid signal count for CEP7 and *MYC* and an aneuploid signal count (five signals) for *TERC*, while nucleus **E** was HPV-FISH negative (there were however HPV-FISH-positive nuclei within the same sample) and showed three signals for CEP7, six signals for *TERC*, and four signals for *MYC*. The aberrant patterns for the latter two nuclei were clonal within these cases (cases 113 and 227).

Statistical Methods

Receiver operating characteristics (ROC) curves were used to establish optimal thresholds and to identify FISH count parameters that best distinguish Thinprep samples with underlying CIN2, CIN3, and cancer histology, from Thinprep samples with underlying WNL and CIN1 histology. ROC curves were generated by plotting the sensitivity for detecting CIN2, CIN3, and cancer versus 1 minus the specificity for detecting WNL and CIN1, obtained at percent cell thresholds ranging from 0% to 10% (0.05% increments). Curves were generated based on the number of tetraploid cells, the number of cells with *TERC* gain (excluding "tetraploid" cells and cells that have four CEP7 signals and four *TERC* signals), the number of cells with either tetraploidy or *TERC* gain (ie, all gains of *TERC*), the number of cells with *MYC* gain (excluding "tetraploid" cells and cells that have four CEP7 signals and four *MYC* signals), and the number of cells with either tetraploidy or *MYC* gain (ie, all gains of *MYC*). Curves that come closest to the ideal values of 100% sensitivity and 100% specificity (Figure 4A, top left corner of ROC graph) provide the best combination of sensitivity and specificity (assuming equal importance of each) and optimal thresholds are typically selected from points near the "breaks" in the curves (region closest to top left corner; curve slope near 45°). A better view of the dependence of sensitivity and specificity on threshold can be obtained by plotting the distance from ideal (DFI) versus threshold (Figure 4B). DFI is defined here

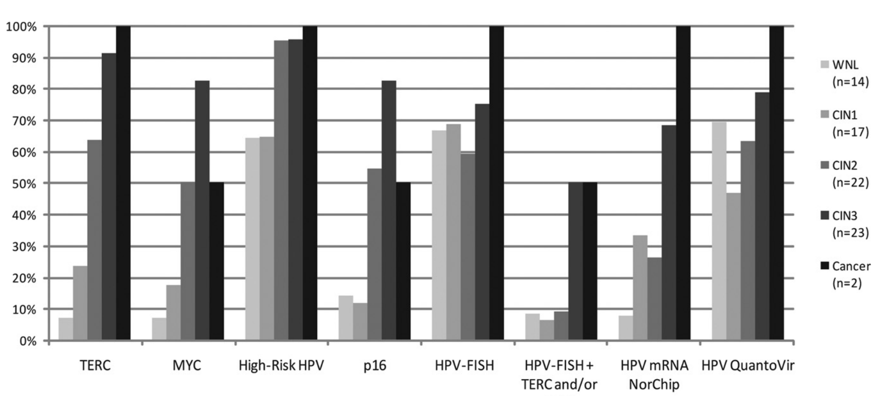


Figure 2. Percentage of positive cases within the categories WNL, CIN1, CIN2, CIN3, and cancer for the different tests applied. Data for HPV Quantovir, Norchip mRNA, and p16 have been obtained from previous publications.[12,18,20]

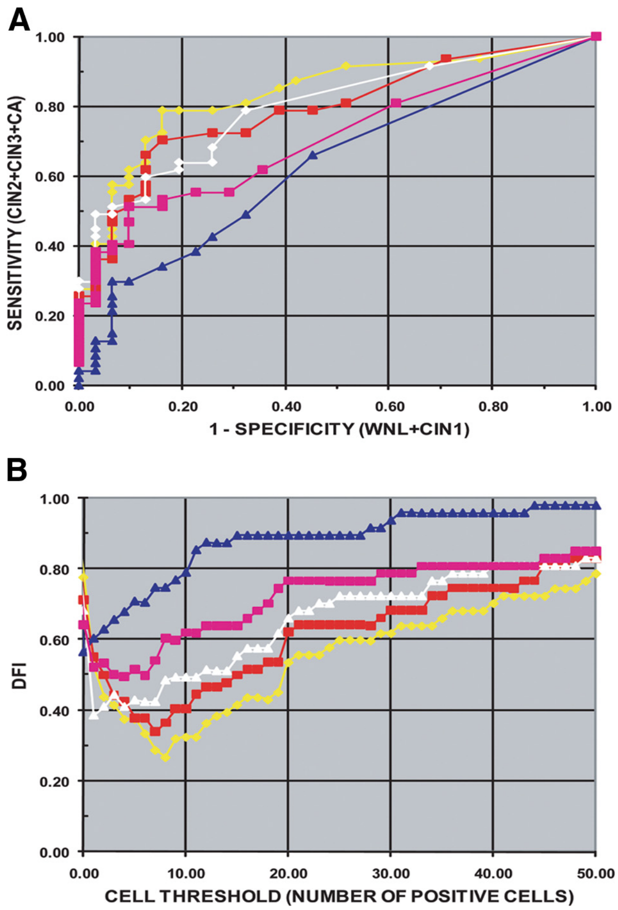


Figure 4. ROC and DFI curves for *TERC* and *MYC* gain. **A:** ROC plot of sensitivity versus 1-specificity at thresholds ranging between 0% and 100% abnormal cells. **B:** Plot of DFI versus threshold (refer to *Materials and Methods* for details). The yellow diamonds in both **A** and **B** show the results when considering cells with any *TERC* gain (>2 *TERC* signals/cell including cells with four signals for CEP7 and *TERC* and "tetraploid" cells); the white diamonds denote the results when considering cells with more than two *TERC* signals as positive, excluding cells with four signals for CEP7 and *TERC* and "tetraploid" cells; the red squares display the results when considering cells with any *MYC* gain (more than two MYC signals/cell including cells with four signals for CEP7 and *MYC* and "tetraploid" cells); the pink squares depict results when considering cells with more than two *MYC* signals, excluding cells with four signals or CEP7 and *MYC* and "tetraploid" cells, and the blue triangles reflect the results when only "tetraploid" cells were considered, ie, hybridization patterns of four signals for each probe (4 CEP7 − 4 *TERC* − 4 *MYC*).

as the distance from the idealpoint (0,1) on the ROC plot (100% sensitivity, 100% specificity), and is calculated as $[(1 - \text{sensitivity})^2 + (1 - \text{specificity})^2]^{1/2}$. DFI is smallest for the best combined sensitivity and specificity (giving equal weight to each) and varies from 0 for thresholds providing 100% sensitivity and 100% specificity, to 1.414 for thresholds providing 0% sensitivity and 0% specificity.

Results

A four-color FISH probe set for detection of CEP7, *TERC* (3q26), *MYC* (8q24), and HPV was hybridized to 78 cervical LBC samples of a Swedish patient cohort. Examples of the FISH analyses are shown in Figure 3. For all of the patients a biopsy was taken at a colposcopy performed at the same time as the LBC sampling, and for most of the patients a subsequent LEEP histology and Pap smear follow-up were available. When no visible lesion was observed, a biopsy was taken close to the squamocolumnar junction.

The LBC samples were also analyzed by line blot and Quantovir for HPV infection, detection of E6/E7 mRNA of HPV 16, 18, 31, 33, and 45 high-risk types of HPV expression with the real-time nucleic acid sequence-based amplification assay and for p16 expression by immunocytochemistry. The study design is presented in Figure 1. Table 1 summarizes the FISH, HPV, and p16 data available for each sample together with the cytologic screening results, the histological diagnoses from biopsy and LEEP, and the clinical follow-up data. The results of the primary Pap smears taken at the population-based screenings at the referral clinics for the 78 women enrolled showed ASCUS in 14 cases, 34 smears had LSIL, 22 had HSIL, and six had ASC-H. In two cases we were unable to retrieve the initial diagnosis.

The samples in Table 1 are arranged by the highest grade of histological diagnosis (when biopsy and LEEP histology differed from each other, the higher grade was used).

When sorting according to the highest grade histology, 14 of the 78 samples were WNL, 17 samples were CIN1, 22 samples were CIN2, 23 samples were CIN3, and two samples were squamous cell cervical carcinoma, maintaining a good balance among the CIN groups. The number of *TERC*-positive cases increased with severity of the cervical lesion. Applying a threshold of more than eight *TERC*-positive cells before a case was considered *TERC* positive, 7% (one of 14) of WNL cases, 24% (4/17) of CIN1, 64% (14/22) of CIN2, 91% (21/23) of CIN3, and 100% (2/2) of the carcinomas were positive for genomic amplification of *TERC*. Figure 2[12,18,20] shows the percentages of positive cases in lesions assessed as WNL, CIN1, CIN2, CIN3, and cancer comparing the different markers and tests used in this study. The *TERC* test achieved the highest combined specificity and sensitivity for discernment of low-grade from high-grade lesions and cancer. This can also be appreciated in Table 2, which shows the sensitivity and specificity for the different tests used. When distinguishing between benign and CIN1 lesions on the one side and CIN2, CIN3, and cancers on the other, the *TERC* test has a sensitivity of 79% and a specificity of 84%. When distinguishing between benign lesions versus CIN3 lesions the sensitivity of the test is 91% and the specificity is 93%. In comparison, as expected from a referral population, the HPV test (line blot) has a very high sensitivity (97% high-risk-HPV, 100% low-risk and high-risk-HPV), but an extremely low specificity (36% HR-HPV, 19% LR and HR-HPV). Expression of p16 and *MYC* copy number are both associated with a slightly better specificity than *TERC,* but their sensitivities are around 10% points lower than the sensitivity of the *TERC* test. Noteworthy, in the distinction of WNL samples versus CIN3 samples, *TERC* performs better both in specificity and sensitivity, with 7% points and 9% points, respectively, as compared with p16. HPV-FISH alone has a low specificity, but also a relatively low sen-

Table 2. Sensitivities and Specificities of the Different Tests Detecting WNL and CIN1 Lesions Versus CIN2, CIN3, and Cancer Lesions and Detecting WNL Versus CIN3 Lesions from a Sample of Women Referred to Colposcopy and Punch Biopsies Based on a Prior Abnormal Pap Smear Result

	WNL and CIN1 versus CIN2, CIN3 and CA		WNL versus CIN3	
	Sensitivity	Specificity	Sensitivity	Specificity
TERC (cut-off nine and more positive nuclei)	78.7%	83.9%	91.3%	92.9%
MYC (cut-off nine and more positive nuclei)	66.0%	87.1%	82.6%	92.9%
High-risk HPV	97.0%	35.5%	95.7%	35.6%
Low-risk and high-risk HPV	100.0%	19.4%	100.0%	14.3%
p16	68.1%	87.1%	82.6%	85.6%
High-risk HPV + p16	68.1%	87.1%	82.6%	85.6%
HPV-FISH (one and more positive nuclei)	68.2%	42.1%	75.0%	43.3%
HPV-FISH + *TERC* and/or *MYC* (four and more double positive nuclei)	27.7%	93.5%	50.0%	91.7%
HPV viral load, Quantovir assay	72.5%	42.9%	78.9%	30.8%
mRNA HPV, NorChip assay	50.0%	78.6%	68.4%	92.3%

sitivity, especially in comparison with the line blot HPV results. This can be mostly attributed to the inability of the HPV-FISH test to detect HPV type 31 in our Swedish material, though the probe has been shown to detect type 31 in other cohorts.[22] The HPV-FISH test did not detect 20 cases that were positive by HPV line blot, of which seven cases were type 31, two cases were type 68, and two cases were type 73. Types 68 and 73 are not expected to be detected by HPV-FISH since there is only 35% homology between these types and the types in the probe mix. The rest of the cases not detected by HPV-FISH involved types 16, 18, 33, 39, 51, 53, 56, and 66. When using a threshold that has been proposed and applied by Sokolova et al[22] (ie, four or more cells positive for both *TERC* and/or *MYC* and HPV-FISH), we achieve good specificity but very low sensitivity (see Table 2), which can be partly explained by the suboptimal performance of the HPV-FISH probe in our material. However, even CIN3 cases that were HPV-FISH positive and had chromosomally aberrant nuclei, did not necessarily have four and more double-positive nuclei and were therefore negative when using this criterion (see cases 181, 240, 227, see Table 1).

Both, the Quantovir and the line blot HPV tests have low specificity (42.9% and 35.5%, respectively), however, the line blot has a higher sensitivity (97% vs. 72.5%). The discrepancy in sensitivity can be partly explained by the fact that the Quantovir test does not cover all HPV types tested for in the line blot assay. Disagreements between types found by line blot and types found by Quantovir are most likely due to binding of the Quantovir primers to HPV types other than the one they were designed for because of sequence homology.

We did not observe a clear increase in the incidence of HR-HPV oncogene mRNA expression with increasing severity of the lesion by using nucleic acid sequence-based amplification assay (NorChip). The HPV mRNA test (NorChip) shows good specificity, but lacks sensitivity (only 50% of CIN2, CIN3, and cancer lesions are detected), which can be mainly attributed to its inability to detect CIN2 lesions. Its sensitivity for detecting CIN3 lesions is better with 68.4%, but still far below the *TERC* marker (91.3%).

Figure 5 demonstrates that the number of *TERC*-positive cells is positively correlated with the severity of the lesions. Interestingly, *TERC*-positive cells often display similar or even identical patterns within the same lesions (Table 1, signal patterns observed), which is a strong indication for expansion of an initial clone following the acquisition of extra copies of 3q. Especially in the more severe lesions, it is evident that the high numbers of *TERC*-positive cells are often due to the expansion of one or a few specific clones. This is exemplified in, eg, case 227, with aberrant nuclei displaying only one pattern, ie, "364" in 131 of 133 cells with aberrant signal counts.

We also analyzed sections of the biopsies and the LEEPs of the two CIN3 cases that were not positive for *TERC* gain in the LBC samples. The LBC samples of both of these cases were scored as LSIL. Case 230 had a CIN3 biopsy and a CIN1 LEEP and case 118 had a CIN1 biopsy and a CIN3 LEEP. The CIN3 biopsy of case 230 showed a small nest of *TERC*-positive cells while the CIN1 LEEP turned out to be negative, indicating that the biopsy very possibly removed the *TERC* positive CIN3 cells completely. The CIN1 biopsy of case 118 was *TERC*-negative, however, the CIN3 LEEP taken 6 months later showed *TERC* positive cells (see Figure 6, A–D).

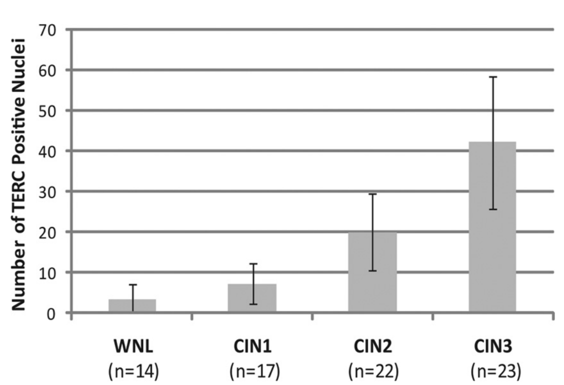


Figure 5. Average number of *TERC*-positive cells observed in WNL, CIN1, CIN 2, and CIN3 lesions. Error bars indicate SEM.

Classification Algorithms

Figure 4A shows ROC curves for discriminating CIN2, CIN3, and cancer, from lesions diagnosed as WNL and CIN1. Based on the curve lying closest to the top left corner of the graph, *TERC* gain including tetraploidy provides the best method for distinguishing CIN2, CIN3, and cancer from WNL and CIN1. Figure 4B shows DFI curves, and as expected from the ROC curves, *TERC* gain including tetraploidy shows the lowest DFI. The minimum of this curve indicates the threshold with the best combined specificity and sensitivity, which in our dataset are 8.6 *TERC*-positive cells. Based on these analyses, we selected the optimal threshold to determine *TERC* positivity as a case with nine and more cells containing more than two signals of *TERC* including "tetraploid" cells and cells with four signals for *TERC* and four signals for CEP7. Using this threshold the *TERC* test reached a specificity of 83.9% and a sensitivity of 78.7% to distinguish benign and CIN1 lesions from CIN2, CIN3, and cancer. The specificity and sensitivity with which WNL could be distinguished from CIN3 was 92.9% and 91.3%, respectively.

Discussion

We have previously demonstrated that a FISH assay for the visualization of genomic amplification of the human telomerase RNA gene *TERC* on chromosome band 3q26.3 can distinguish Pap smears with high-grade cytology from those with normal or low-grade cytology with high sensitivity and specificity.[24] In a second retrospective study, we have expanded on these results by showing that detection of *TERC* gain can help stratifying women with abnormal Pap smears according to the risk of progression from low-grade lesions to higher-grade lesions and cancer. In addition, applying the *TERC* test to routinely collected Pap smears increased the sensitivity of cytological screening.[11]

In the current study, we were able to determine the presence of *TERC*-positive cells in LBC samples collected on the same day as the diagnostic biopsy. In addition, we were able to correlate the *TERC*-positive LBC results with follow-up Pap, LEEP, and/or biopsy samples. By using all available histopathological data from baseline and follow-up, we were able to enhance the diagnostic accuracy of our case definition by defining case status based on the highest grade histology results observed. For example, combining histology results of the biopsy and LEEP has the advantage that possible sampling errors will be reduced, especially as the LEEP excision often results in larger, more representative material. However, having the LEEP excision taken at a later time point can, in certain cases, mean that the lesion advanced to a higher degree than it was actually at the LBC sampling. In contrast, the diagnostic biopsy might have presented a cure with complete removal of dysplastic cells.

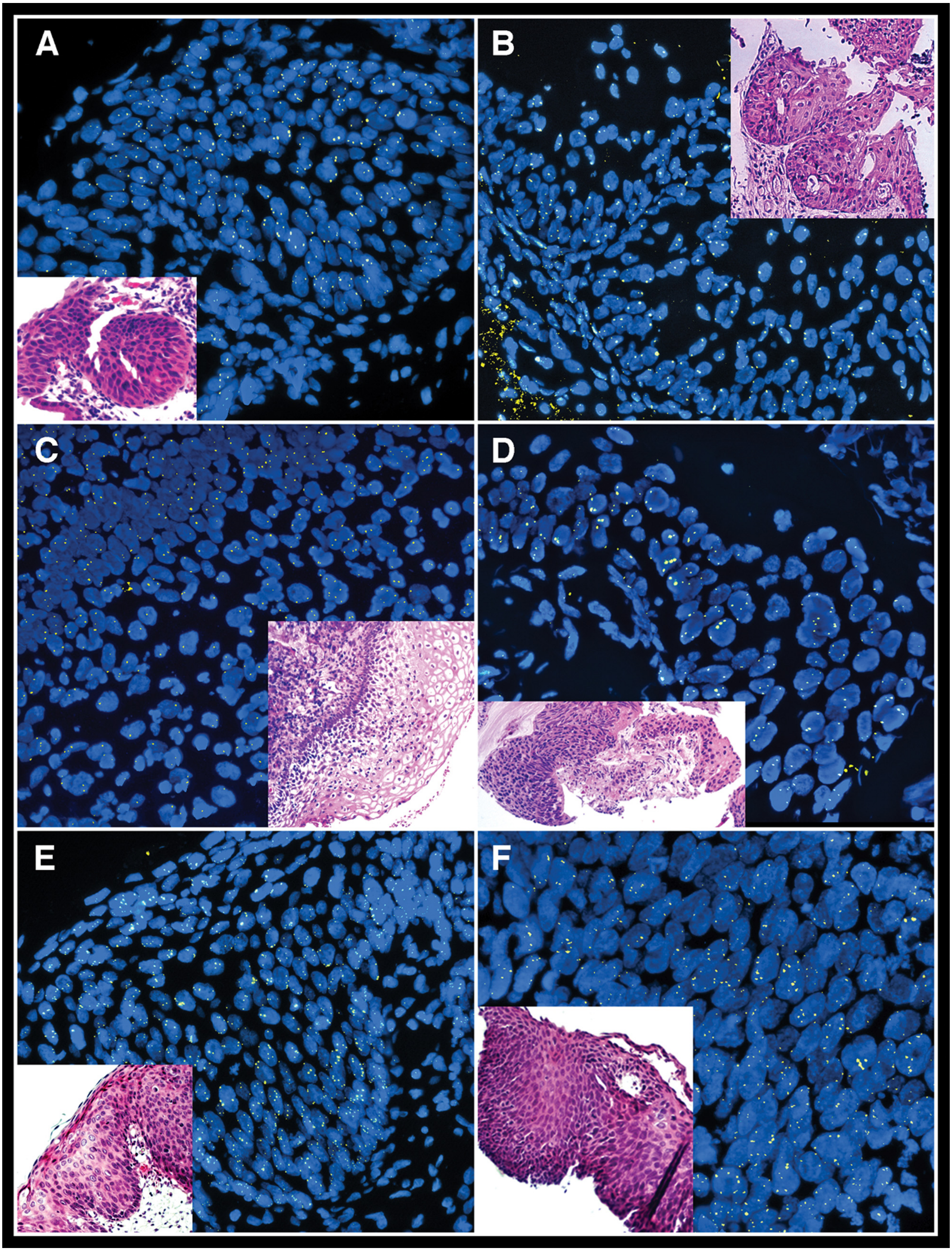
In this study, we also included a *MYC* probe, a CEP7 probe that served as a control for the ploidy of the cells, and a probe for the detection of HPV genomes in the FISH analysis, allowing the evaluation of test performance using *TERC* alone and in combination with these markers.

According to these evaluation parameters, *TERC* positivity was observed in 7% (one of 14) WNL cases, 24% (4/17) CIN1, 64% (14/22) CIN2, 91% (21/23) CIN3 lesions, and 100% (2/2) carcinomas. Interestingly, the two *TERC*-negative cases with CIN3 histopathology (cases 118 and 230, Table 1), had cytological screening results and histological diagnoses of the biopsy and LEEP that differed from each other. The LBC cytology of both cases was assessed as LSIL. Case 118 presented with a CIN1 biopsy and a CIN3 LEEP 6 months later. *TERC* analysis on the histological samples showed negativity for the biopsy and positivity for the LEEP, indicating that the *TERC* gain was not present at the time of the LBC sampling, but developed later during the progression from CIN1 to CIN3 (see Figure 6C, D).

In contrast, case 230 displayed a CIN3 biopsy, while the subsequent LEEP was diagnosed as a CIN1 lesion. *TERC* analysis of these samples showed positivity for the CIN3 biopsy and negativity for the CIN1 LEEP. This case might present an example for the scenario that the diagnostic biopsy can be curative with complete removal of all dysplastic cells. This is further supported by the observation that the area of *TERC*-positive cells within the biopsy was very small and contained (see Figure 6A, B). The small size of the lesion is most likely also the reason that only very few *TERC*-positive cells were detected in the LBC sample (ie, four, which is below the threshold of more than eight positive cells). Interestingly, the original referral Pap smear was judged as ASC-H, indicating that this first cytology sampling possibly yielded a more representative material than the sampling for the LBC. Examples like this one underscore the importance of representative sampling, but also highlight one of the recognized limitations of cervical screening.

The hybridization results of the tissue sections of these two CIN3 cases show that so far we have not observed a cervical lesion with a histological diagnosis greater than CIN2 that does not show TERC positivity. There are, however, *TERC*-negative lesions diagnosed histologically as CIN2. This corroborates that CIN3 can be considered the true endpoint lesion while CIN2 lesions rather present one link in the continuous progression chain that can be further stratified by *TERC* positivity as being associated with significant risk of becoming a CIN3 lesion with further progression to invasive disease.

The diagnostic potential of genomic amplification of *TERC* as a powerful biomarker is further emphasized by the following cases: Case 201 had LBC LSIL cytology with underlying CIN3 histology and was clearly detected with 51 *TERC*-positive cells in the LBC sample. Case 204 cytology was recorded as ASC-H, with 25 *TERC*-positive cells, but the biopsy result only recorded CIN1. However, the LEEP histology 1 month later showed a CIN3 lesion. Case 156 scored normal for cytology, however, we found 20 *TERC*-positive cells. The biopsy was classified as CIN3, and a subsequent LEEP excision 2.5 months later revealed a microinvasive carcinoma. The cytological samples of case 112 were initially assessed as LSIL, which was confirmed in the biopsy, but the LBC sample was positive for *TERC*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amplification. LEEP excision as much as 17 months later revealed a CIN3. Arguably, it is not possible to establish whether a sampling error during the biopsy precluded the detection or, if that is not the case, how soon after the biopsy sampling the lesion progressed to CIN3. However, this case shows the full potential of the *TERC* test, as this patient could have been treated according to the true severity of the lesion before further progression.

It is also interesting to mention case 182, which presented with a HSIL LBC cytology that was strongly positive for *TERC* and *MYC*. However, biopsy, subsequent LEEP and follow-up Pap smears were all benign. Looking at the main pattern of aberration, the *TERC* gene presents with four copies on a tetraploid background, while the *MYC* gene shows a relative gain with five copies. We have observed in a previous study that cases that were positive for *TERC* as a consequence of tetraploidy can either progress or regress.[11] Case 182 seems to be an example for a case that regressed. Interestingly, the *MYC* gene presented a relative gain within the tetraploid nucleus. If this case truly regressed by itself, this would indicate that relative gains for the *MYC* gene are less specific for cervical cancer progression than relative gains for the *TERC* gene, which would be consistent with the fact that gain of chromosome 8q and with it genomic amplification of the *MYC* oncogene is rarely observed in cervical tumorigenesis.

The LBC samples were also analyzed for alternative disease markers including high-risk and low-risk HPV types by HPV line blot, HPV viral load by the Quantovir assay, HPV mRNA expression by nucleic acid sequence-based amplification assay, and p16 expression using immunocytochemistry.

When comparing the different assays used in this study, the line blot HPV test had the best sensitivity, detecting all CIN2 and CIN3 lesions when including both low-risk and high-risk HPV infections. These data corroborate that HPV testing is an excellent first screen to identify women with a higher risk of developing cervical cancer. However, as known before and observed also in this study with a specificity of the line blot test of 19.4% (low-risk and high-risk HPV) or 35.5% (high-risk HPV only), HPV testing has only limited power to stratify low-grade from high-grade disease and can therefore not be used to efficiently triage patients further. Therefore additional markers that can triage patients to avoid overtreatment and not to overlook relevant lesions are needed. Potential triage markers tested in this study are *TERC*, *MYC*, p16, and HPV mRNA expression.

With a specificity of 87.1%, which is slightly better than the *TERC* test (83.9%) and the HPV mRNA expression test (78.7%), p16 and *MYC* performed well. However, their sensitivities with 68.1% and 66.0%, respectively, are more than 10% points lower than the sensitivity of the *TERC* test (78.7%). In the stratification between benign lesions and CIN3 lesions (rather than benign, CIN1, and CIN2 versus CIN3 and cancer), the *TERC* test sensitivity and specificity increased to 91.3% and 92.9%, respectively. The sensitivity of p16 and *MYC* for that stratification increased only to 82.6%, which is 10% lower than the *TERC* test. HPV mRNA showed a very low sensitivity of 50% for the distinction between benign and CIN1 vs. CIN2, CIN3, and cancer, and a slightly better sensitivity of 68.4% for the distinction between benign versus CIN3 lesions. These data show that the *TERC* test has the best combined specificity and sensitivity in the panel of markers tested in this study.

p16 is a cyclin-dependent kinase inhibitor involved in cell cycle control. Current data suggest that inactivation of pRB through HPV E7 results in enhanced expression of p16, which might therefore represent a specific and sensitive biomarker for cells with active expression of HPV oncogenes.[25,26] Several investigators applied p16 as an immunochemical assay to Pap smears, LBC slides or histological samples of benign, dysplastic, and cancerous lesions. Klaes et al[7] reported that all benign lesions and all CIN1 lesions associated with low-risk HPV had no detectable expression of p16, while all CIN2 and CIN3 lesions and all CIN1 lesions with high-risk HPV showed marked overexpression. Other papers show a less stratified and less sensitive outcome, often observing a few positive lesions in the normal and CIN1 entities, and detecting around 70% to 80% of the high-grade lesions.[27–29] These latter observations are more consistent with our data that show few positive lesions in the WNL and CIN1 category, and 55% and 83% positivity for CIN2 and CIN3 lesions, respectively. Interestingly, p16 was not positive in one of the two cancers in our material. P16-negative cervical cancers have been previously observed by other investigators, and Masoudi et al[30] speculate that the decrease or negativity in some of the carcinomas could be due to a loss of expression during progression, perhaps as a result of a promoter methylation. Interestingly, they found that p16 negativity in squamous cell carcinoma was an adverse prognostic marker.

Recently, two other studies applying the *TERC* FISH probe as a diagnostic marker on cervical Thinprep samples were published.[22,31] The studies are similar in that they reduce the amount of technician time required to evaluate the specimens. Both studies are in agreement with our data that *TERC* positivity can detect high-grade lesions with high sensitivity. However, both studies observe a rather low specificity of the test, in contrast to our findings. On careful evaluation, the possible explanation

Figure 6. Histopathology and corresponding FISH images (*TERC* marker in yellow) of the biopsies and LEEPs of cases 118, 230, 250, and 90. **A:** Biopsy 230 (CIN3) shows several nuclei with more than three *TERC* signals. However, the corresponding LBC sample was below the *TERC*-positive threshold (see Table 1), possibly because of sampling error or too few aberrant cells present in the lesion. **B:** The LEEP 230 taken 1 month later only shows histology grade CIN1 and does not harbor any nuclei with more than two *TERC* signals, indicating that the biopsy might have eradicated the TERC positive lesion completely. **C:** Biopsy 118 (CIN1) does not show any nuclei with more than two *TERC* signals, which is in agreement with the result of the LBC sample (Table 1). **D:** LEEP 118, taken 6 months after the biopsy, was diagnosed as a CIN3 and showed several nuclei with more than three *TERC* signals indicating that the *TERC* gain developed during the progression of the lesion. **E:** LEEP 250 (CIN3) showed many nuclei with three and more signals for *TERC* throughout the epithelium, which is in concordance with the aberrations found in the LBC sample (Table 1). **F:** Biopsy 90 (CIN3) also displayed many nuclei with more than three signals for *TERC* (main pattern five *TERC* signals), which correlates well with the more severe main pattern found in the LBC sample (ie, two signals for CEP7, five signals for *TERC*, two signals for *MYC*, Table 1).

for this discrepancy seems to be mostly due to different evaluation methods. Caraway et al[31] applied an automated evaluation approach based on a commercial imaging system (MetaSystems, Waltham, MA) and on an algorithm similar to the one used for automated evaluation of UroVysion samples.[32] The enumeration strategy included a pre-selection of cells of interest based on nuclear size and shape. They also drastically reduced the number of evaluated nuclei to only the 25 most atypical nuclei per slide. In this approach, the threshold was put to two and more cells with more than two *TERC* signals and all cytological HSIL lesions were detected (100% sensitivity), which equals our results. However, due to the low threshold, also 70% of the LSIL lesions were scored as positive. Another drawback of this method is that *TERC* aberrations do not always happen in the largest and most irregular nuclei, but do present in morphologically unsuspicious nuclei.[11]

Sokolova et al[22] used a combined evaluation approach of *TERC* and/or *MYC* positivity within HPV-FISH−positive cells with a threshold of four and more double-positive cells. By this method, the specimen is scanned for HPV-stained cells and only those cells are enumerated. In the absence of HPV staining a representative number of cells are enumerated. The immediate drawback that the authors experienced with this approach is that not all *TERC*-positive cells show HPV-FISH positivity, partly because of sensitivity issues of the HPV-FISH test, and partly because of the possibility that cells that have acquired chromosomal aneuploidies can become negative for HPV. The authors mention that two of their high-grade lesions show a substantial number of *TERC*-positive cells, but cannot be scored positively, due to the fact that these cells are not positive for HPV-FISH, which compromises the sensitivity of the TERC test to a certain degree. Notably, also the specificity of their study is much lower than in our study. Again, it is very likely that the combined evaluation of HPV and *TERC* and *MYC* data within the same cell is responsible for this decrease in specificity. Most LSIL lesions are HPV positive, which is the reason why HPV testing has a very low specificity. If one combines HPV positivity with a relatively low threshold for *TERC*-positive cells (four cells and more), the stratification power of the *TERC* test will be diminished. If we were to apply a threshold of four or more *TERC* and/or *MYC*-positive cells (irrespective of HPV-FISH) to our dataset, we would achieve very similar specificity values as Sokolova et al, [22] ie, 53% (compared with 56%) for CIN1 lesions while increasing the sensitivity to 96% (compared with 86%). This could be interpreted as evidence that HPV-FISH does not contribute specificity to the *TERC* data while reducing sensitivity, although this approach may permit a more rapid means of specimen evaluation that would be beneficial in a routine screening application.

In summary, our data show that the *TERC* test is the most sensitive and specific test for the stratification of benign and low-grade cervical lesions from high-grade lesions and cancer when compared with other tests used for stratification. It also becomes clearly evident that the evaluation method used for genetic FISH markers like the *TERC* test needs to be adapted to the clinical samples analyzed. As screening entire LBC slides for FISH markers is tedious and time-consuming, alternative approaches, such as the two approaches referred to above, should be tested. While it would be desirable to combine FISH markers with tests more easily amenable to a high throughput format, our study shows that such combinations often diluted the performance of detecting genomic amplification of *TERC* as a powerful biomarker for disease progression. These results are supported by a large body of literature demonstrating the central role of this specific aneuploidy for cervical tumorigenesis.[9–11,22,24,31,33–41]

HPV testing is increasingly used as a screening method, either alone or in conjunction with Pap smear testing and its high sensitivity and its ease and reproducibility of analysis makes it a very attractive first screen.[6] However, especially in younger women, HPV infections are often transient and overtreatment of these women is a serious problem. Our study showed that *TERC* is a powerful marker to discern histologically confirmed low-grade from high-grade cervical disease. In the future, one could envision a screening strategy in which HPV-positive Pap smears are triaged with the help of the *TERC* marker that would contribute to a more evidence-based individualized clinical management. This strategy is supported through biological causality, because both HPV and gain of 3q act in concert in disease initiation and progression.

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References

1. World Health Organization: Comprehensive cervical cancer control: a guide to essential practice. Geneva, Switzerland, WHO, 2006, p 3
2. Koss LG: The Papanicolaou test for cervical cancer detection. A triumph and a tragedy. JAMA 1989, 261:737–743
3. Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Munoz N: Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 1999, 189:12–19
4. Khan MJ, Castle PE, Lorincz AT, Wacholder S, Sherman M, Scott DR, Rush BB, Glass AG, Schiffman M: The elevated 10-year risk of cervical precancer and cancer in women with human papillomavirus (HPV) type 16 or 18 and the possible utility of type-specific HPV testing in clinical practice. J Natl Cancer Inst 2005, 97:1072–1079
5. Cuzick J, Arbyn M, Sankaranarayanan R, Tsu V, Ronco G, Mayrand MH, Dillner J, Meijer CJ: Overview of human papillomavirus-based and other novel options for cervical cancer screening in developed and developing countries. Vaccine 2008, 26 Suppl 10:K29−K41
6. Gravitt PE, Coutlee F, Iftner T, Sellors JW, Quint WG, Wheeler CM: New technologies in cervical cancer screening. Vaccine 2008, 26 Suppl 10:K42−K52
7. Klaes R, Friedrich T, Spitkovsky D, Ridder R, Rudy W, Petry U, Dallenbach-Hellweg G, Schmidt D, von Knebel Doeberitz M: Overexpression of p16(INK4A) as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. Int J Cancer 2001, 92:276–284
8. Norman I, Brismar S, Zhu J, Gaberi V, Hagmar B, Hjerpe A, Andersson

S: p16(INK4a) immunocytochemistry in liquid-based cervical cytology: is it feasible for clinical use? Int J Oncol 2007, 31:1339–1343

9. Heselmeyer K, Schrock E, du Manoir S, Blegen H, Shah K, Steinbeck R, Auer G, Ried T: Gain of chromosome 3q defines the transition from severe dysplasia to invasive carcinoma of the uterine cervix. Proc Natl Acad Sci USA 1996, 93:479–484
10. Heselmeyer K, Macville M, Schrock E, Blegen H, Hellstrom AC, Shah K, Auer G, Ried T: Advanced-stage cervical carcinomas are defined by a recurrent pattern of chromosomal aberrations revealing high genetic instability and a consistent gain of chromosome arm 3q. Genes Chromosomes Cancer 1997, 19:233–240
11. Heselmeyer-Haddad K, Sommerfeld K, White NM, Chaudhri N, Morrison LE, Palanisamy N, Wang ZY, Auer G, Steinberg W, Ried T: Genomic amplification of the human telomerase gene (TERC) in pap smears predicts the development of cervical cancer. Am J Pathol 2005, 166:1229–1238
12. Andersson S, Hansson B, Norman I, Gaberi V, Mints M, Hjerpe A, Karlsen F, Johansson B: Expression of E6/E7 mRNA from 'high risk' human papillomavirus in relation to CIN grade, viral load and p16INK4a. Int J Oncol 2006, 29:705–711
13. Solomon D, Davey D, Kurman R, Moriarty A, O'Connor D, Prey M, Raab S, Sherman M, Wilbur D, Wright T, Jr., Young N: The 2001 Bethesda System: terminology for reporting results of cervical cytology. JAMA 2002, 287:2114–2119
14. Richart RM: A modified terminology for cervical intraepithelial neoplasia. Obstet Gynecol 1990, 75:131–133
15. Peyton CL, Gravitt PE, Hunt WC, Hundley RS, Zhao M, Apple RJ, Wheeler CM: Determinants of genital human papillomavirus detection in a US population. J Infect Dis 2001, 183:1554–1564
16. Gravitt PE, Peyton CL, Alessi TQ, Wheeler CM, Coutlee F, Hildesheim A, Schiffman MH, Scott DR, Apple RJ: Improved amplification of genital human papillomaviruses. J Clin Microbiol 2000, 38:357–361
17. Gravitt PE, Peyton CL, Apple RJ, Wheeler CM: Genotyping of 27 human papillomavirus types by using L1 consensus PCR products by a single-hybridization, reverse line blot detection method. J Clin Microbiol 1998, 36:3020–3027
18. Andersson S, Safari H, Mints M, Lewensohn-Fuchs I, Gyllensten U, Johansson B: Type distribution, viral load and integration status of high-risk human papillomaviruses in pre-stages of cervical cancer (CIN). Br J Cancer 2005, 92:2195–2200
19. Moberg M, Gustavsson I, Gyllensten U: Real-time PCR-based system for simultaneous quantification of human papillomavirus types associated with high risk of cervical cancer. J Clin Microbiol 2003, 41:3221–3228
20. Andersson S, Wangsa D, Flores-Staino C, Safari H, Mints M, Hjerpe A, Hagmar B, Johansson B: Expression of p16(INK4a) in relation to histopathology and viral load of 'high-risk' HPV types in cervical neoplastic lesions. Eur J Cancer 2006, 42:2815–2820
21. Linder J, Zahniser D: ThinPrep Papanicolaou testing to reduce false-negative cervical cytology. Arch Pathol Lab Med 1998, 122:139–144
22. Sokolova I, Algeciras-Schimnich A, Song M, Sitailo S, Policht F, Kipp BR, Voss JS, Halling KC, Ruth A, King W, Underwood D, Brainard J, Morrison L: Chromosomal biomarkers for detection of human papillomavirus associated genomic instability in epithelial cells of cervical cytology specimens. J Mol Diagn 2007, 9:604–611
23. Algeciras-Schimnich A, Policht F, Sitailo S, Song M, Morrison L, Sokolova I: Evaluation of quantity and staining pattern of human papillomavirus (HPV)-infected epithelial cells in thin-layer cervical specimens using optimized HPV-CARD assay. Cancer 2007, 111:330–338
24. Heselmeyer-Haddad K, Janz V, Castle PE, Chaudhri N, White N, Wilber K, Morrison LE, Auer G, Burroughs FH, Sherman ME, Ried T: Detection of genomic amplification of the human telomerase gene (TERC) in cytologic specimens as a genetic test for the diagnosis of cervical dysplasia. Am J Pathol 2003, 163:1405–1416
25. Keating JT, Ince T, Crum CP: Surrogate biomarkers of HPV infection in cervical neoplasia screening and diagnosis. Adv Anat Pathol 2001, 8:83–92
26. Wang JL, Zheng BY, Li XD, Angstrom T, Lindstrom MS, Wallin KL: Predictive significance of the alterations of p16INK4A, p14ARF, p53, and proliferating cell nuclear antigen expression in the progression of cervical cancer. Clin Cancer Res 2004, 10:2407–2414
27. Meyer JL, Hanlon DW, Andersen BT, Rasmussen OF, Bisgaard K: Evaluation of p16INK4a expression in ThinPrep cervical specimens with the CINtec p16INK4a assay: correlation with biopsy follow-up results. Cancer 2007, 111:83–92
28. Duncan L, Jacob S, Hubbard E: Evaluation of p16INK4a as a diagnostic tool in the triage of Pap smears demonstrating atypical squamous cells of undetermined significance. Cancer 2008, 114:34–48
29. Zhang Q, Kuhn L, Denny LA, De Souza M, Taylor S, Wright TC Jr: Impact of utilizing p16INK4A immunohistochemistry on estimated performance of three cervical cancer screening tests. Int J Cancer 2007, 120:351–356
30. Masoudi H, Van Niekerk DJ, Gilks CB, Cheang M, Bilek K, Fischer U, Ehlen T, Miller D, Horn LC: Loss of p16 INK4 expression in invasive squamous cell carcinoma of the uterine cervix is an adverse prognostic marker. Histopathology 2006, 49:542–545
31. Caraway NP, Khanna A, Dawlett M, Guo M, Guo N, Lin E, Katz RL: Gain of the 3q26 region in cervicovaginal liquid-based pap preparations is associated with squamous intraepithelial lesions and squamous cell carcinoma. Gynecol Oncol 2008, 110:37–42
32. Sokolova IA, Halling KC, Jenkins RB, Burkhardt HM, Meyer RG, Seelig SA, King W: The development of a multitarget, multicolor fluorescence in situ hybridization assay for the detection of urothelial carcinoma in urine. J Mol Diagn 2000, 2:116–123
33. Kirchhoff M, Rose H, Petersen BL, Maahr J, Gerdes T, Lundsteen C, Bryndorf T, Kryger-Baggesen N, Christensen L, Engelholm SA, Philip J: Comparative genomic hybridization reveals a recurrent pattern of chromosomal aberrations in severe dysplasia/carcinoma in situ of the cervix and in advanced-stage cervical carcinoma. Genes Chromosomes Cancer 1999, 24:144–150
34. Hopman AH, Theelen W, Hommelberg PP, Kamps MA, Herrington CS, Morrison LE, Speel EJ, Smedts F, Ramaekers FC: Genomic integration of oncogenic HPV and gain of the human telomerase gene TERC at 3q26 are strongly associated events in the progression of uterine cervical dysplasia to invasive cancer. J Pathol 2006, 210:412–419
35. Rao PH, Arias-Pulido H, Lu XY, Harris CP, Vargas H, Zhang FF, Narayan G, Schneider A, Terry MB, Murty VV: Chromosomal amplifications, 3q gain and deletions of 2q33–q37 are the frequent genetic changes in cervical carcinoma. BMC Cancer 2004, 4:5
36. Matthews CP, Shera KA, McDougall JK: Genomic changes and HPV type in cervical carcinoma. Proc Soc Exp Biol Med 2000, 223:316–321
37. Dellas A, Torhorst J, Jiang F, Proffitt J, Schultheiss E, Holzgreve W, Sauter G, Mihatsch MJ, Moch H: Prognostic value of genomic alterations in invasive cervical squamous cell carcinoma of clinical stage IB detected by comparative genomic hybridization. Cancer Res 1999, 59:3475–3479
38. Allen DG, White DJ, Hutchins AM, Scurry JP, Tabrizi SN, Garland SM, Armes JE: Progressive genetic aberrations detected by comparative genomic hybridization in squamous cell cervical cancer. Br J Cancer 2000, 83:1659–1663
39. Umayahara K, Numa F, Suehiro Y, Sakata A, Nawata S, Ogata H, Suminami Y, Sakamoto M, Sasaki K, Kato H: Comparative genomic hybridization detects genetic alterations during early stages of cervical cancer progression. Genes Chromosomes Cancer 2002, 33:98–102
40. Hidalgo A, Monroy A, Arana RM, Taja L, Vazquez G, Salcedo M: Chromosomal imbalances in four new uterine cervix carcinoma derived cell lines. BMC Cancer 2003, 3:8
41. Narayan G, Pulido HA, Koul S, Lu XY, Harris CP, Yeh YA, Vargas H, Posso H, Terry MB, Gissmann L, Schneider A, Mansukhani M, Rao PH, Murty VV: Genetic analysis identifies putative tumor suppressor sites at 2q35–q36.1 and 2q36.3-q37.1 involved in cervical cancer progression. Oncogene 2003, 22:3489–3499